

Early and Delayed Cellular Responses in Human Lymphocytes Following Ultra-Violet Exposure

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ABSTRACT: UV-B radiation has roles in DNA damage and cancer induction in skin cells. As well as UV radiation has the ability to penetrate skin reaches to peripheral blood. Therefore, we are here to investigate the initial and delayed effects of range of UV-B irradiation doses (5, 10, 15, 20 and 25 J/cm²), utilizing human lymphocytes. Cells were irradiated and subjected to cell viability test, comet assay, apoptotic and chromosomal analyses at first and 12 generations for early and late responses respectively. Irradiated cells demonstrated initial cellular responses in terms of viability test, comet assay, apoptosis and chromosome aberrations. Alternatively, delayed cell death was observed only following 5 J/cm². Moreover, delayed comet data was insignificant in all experimental groups. Whilst genomic instability was observed after 12 population doublings post 10, 15, 20 and 25 J/cm² UV irradiation in terms of chromosome aberrations. However, 5 J/cm² UV irradiation caused a delayed induction of apoptosis, but not chromosomal instability. Data suggested that exposure to higher than 5 J/cm² UV radiation can instigate genomic instability, which has been considered a hallmark of carcinogenesis. The results also suggested there is an inverse relationship between apoptosis and chromosomal instability in human lymphocytes following UV irradiation.

KEYWORDS: DNA, UV irradiation, Cancer, human Lymphocytes, Chromosomal Analysis.

I. INTRODUCTION

Ultraviolet light (UV) is a non-ionising electromagnetic radiation with wavelength from 200 to 400 nm. According to wavelength, UV is classified into 3 spectra: UVC, which is the shortest spectrum with wavelength 200 to 280 nm. UVB, with 280-315 nm, and UVA, which is about 280- 400 nm. Generally, UV has a great potential risk to induce biological damage in the living tissue [1]. UVC is considered the most dangerous UV spectrum in terms of causing DNA damage. However, the ozone layer interrupts UVC from reaching the earth. The other spectra (UVB and A) can pass to earth through ozone layer [2]. Both of them can cause photo-oxidation damage in the guanine and pyrimidine, instigating DNA damage [3].

UVA and UVB can induce oxidative stress and reactive oxygen species (ROS) [4]. Thus can lead to chromosomal aberrations and cellular damage [5]. Moreover, much evidence has demonstrated that UV frequently cause several DNA damage through signaling cascades, such as ATR/Chk1, and p53 kinase pathways, instigating apoptotic induction and senescence [6]. Furthermore, study has documented that UV can induce bystander effects (BE), which is the biological effects within the unirradiated cells that are in vicinity of irradiated cells, within human endothelial cells [4]. However, UV-induced genomic instability (GI) has not been reported to be one of UV effects. Much evidence has stated that GI is the delayed damage responses within the progeny of irradiated and bystander cells following irradiation. As well as GI is considered the hallmark of carcinogenesis [7]. Therefore, this study was established to estimate UVB-induced genomic instability in human lymphocytes.

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II. MATERIALS AND METHODS

Cell Culture

Human lymphocyte cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum, (FBS, Sigma); 2 mM L-Glutamine (Gibco) and 1% (v/ v) penicillin/streptomycin solution (Sigma) in a humidified 5% CO₂ incubator at 37°C.

UV irradiation

Cells were grown in 96-well plates for viability test, and in T25 flasks for other biological end points (apoptosis and chromosomal analysis). Cells were exposed to 0 J/cm², 5 J/cm², 10 J/cm², 15 J/cm², 20 J/cm², and 25 J/cm². Then cells were subjected to viability assay, apoptotic and chromosomal analyses during the first population doubling following UV light exposure. Another set of experimental groups was propagated up to 20 population doublings to estimate the delayed cellular responses; then cells were analysed for viability, apoptosis and chromosomal instability.

Viability test

Cells were seeded in 96-well plates in 5000 cells per well. The 96-well plates were irradiated with 0 J/cm², 5 J/cm², 10 J/cm², 15 J/cm², 20 J/cm², and 25 J/cm² UV after 24 hours incubation. Cells were then treated with 10 µl alamar blue (Sigma- Aldrich) per each well, and then cells were incubated for 24 hours. Then cell density was measured using plate reader at 540-570 nm.

Comet Assay

Single-cell gel electrophoresis or comet assay is a simple and sensitive method to quantify total DNA damage (double-strand breaks, single-strand breaks and base damage) in individual cells [8, 9]. The comet assay was performed as described [10, 11]. Briefly, microscope slides were coated with 1% normal melting point agarose (NMPA) and allowed to dry overnight. The coated slides were then placed on a metal tray on ice. Ten thousand cells were re-suspended with 200 µl of 1% low melting point agarose (LMPA) and placed immediately onto chilled pre-coated slides. The cell-LMPA suspensions were flattened with cover slips, which were removed after 5 min. The slides were then transferred to a jar, which was filled with cold lysis buffer (2.5 M NaCl, 100 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.6, and 1% Triton X-100, pH. 10). The jar was kept at 4 °C overnight. The slides were then moved to a horizontal electrophoresis tank filled with electrophoresis buffer (0.3M NaOH and 1mM EDTA, pH13) at 4 °C. The electrophoresis was run for 30 min, at 19 V, 300 A. A human lymphocytes nuclei assay were taken, for comparison, it has been taken two human lymphocytes nuclei undamaged and damaged one. In figure 1 A, one could see the undamaged human lymphocytes nucleus while B shows the damaged one as clearly seen from the small head or the remaining part of nucleus.

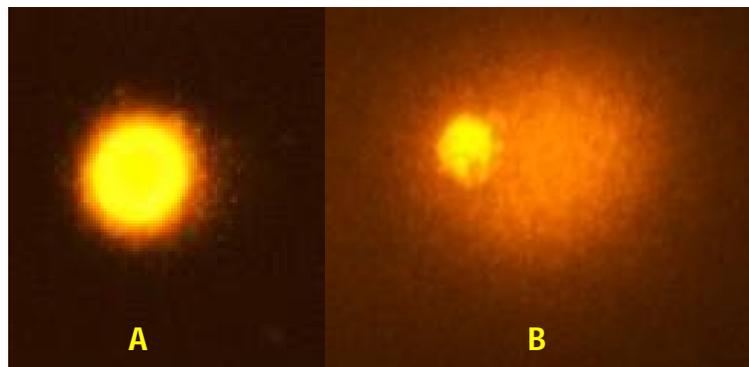


Fig. 1: Human lymphocytes nuclei in comet assay images. Panel A shows undamaged lymphocyte nucleus; whilst B demonstrates damaged nucleus, which has a small head or the remaining part of nucleus (undamaged DNA) and large tail, which represents damaged DNA that left the nucleus.

Slides were neutralised with neutralising buffer (0.4 M Tris-HCl, pH 7.5), washed with distilled water, and immediately stained with a 1:10,000 dilution of SYBR Gold (Invitrogen). The slides were analysed using CASP Software (www.casp.of.pl).

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Chromosomal Analysis

Cells were harvested for metaphase preparation using the established method [12] (26). Briefly, cells were treated with 20 ng/ml demecolcine (Sigma) for 1.5 h in a humidified 5% CO₂ incubator at 37 °C. Cells were centrifuged at 259g for 10 min. The supernatant was discarded, while the cell pellet was re-suspended with a hypotonic solution (75 mM potassium chloride solution (KCl, Sigma) for 20 min at 37°C). The hypotonic cell suspensions were centrifuged at 180g for 10 min. The supernatant was aspirated, and the cell pellet was fixed twice with 25% acetic acid in methanol. Fixed cells were then dropped onto clean slides, and stained using the Giemsa solid staining technique. At least 100 metaphases were analysed per group.

Apoptotic analysis

Apoptotic estimating method has been described by Schwart and co-workers [13]. Briefly, cells were cultured in T25 tissue culture flasks. Then Cells were collected into universal tubes and washed twice with 25% acetic acid in methanol post irradiation. Cells were dropped onto slides and stained with prolong DAPI (Invitrogen). The normal cell nucleus uniformly stains with DAPI, whilst the apoptotic cell nucleus shows apoptotic bodies using fluorescent microscope. At least 1000 cells per each experimental group were analysed. Figure 2 shows a comparison between undamaged or pre UV irradiation as one can see from the normal nucleus on the left hand of Fig.2. However, the right hand image shows the apoptotic nuclei which are damaged due to the fact they have been exposed to the UV light.

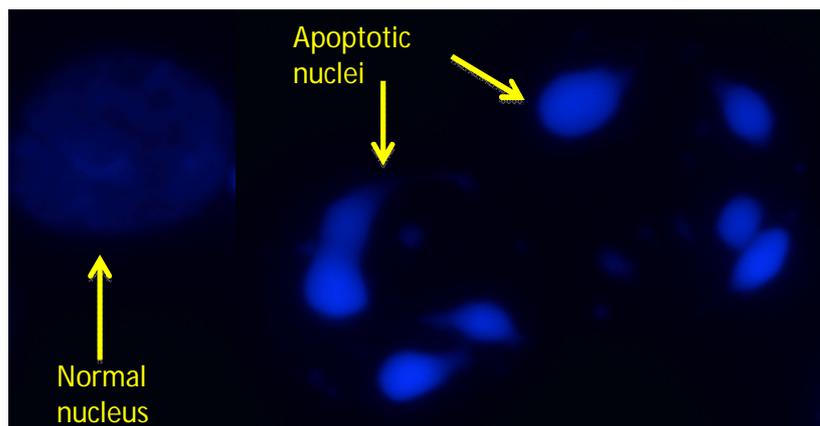


Fig. 2: Lymphocyte nuclei, which shows undamaged (normal nucleus) that uniformly stained with DAPI (left-hand image). Alternatively, right-hand image demonstrates apoptotic nuclei (bodies) following UV light exposure.

Statistical Analyses

For viability test and apoptotic analysis, student *t* test were utilised to measure the significant differences between experimental groups. At least 200 cells per group were analysed analysis of comet assays; statistical analysis was performed using the Mann-Whitney Test, utilising the median of raw data. The Mann-Whitney test deals with the median of nonparametric data. Chromosomal data, represented as mean aberrations per cell, was subjected to the Mann-Whitney test. For each group, 100 metaphases were analysed. The 0.5 *p*-value or less was statistically considered significant difference between compared groups. Each experiment was carried out 3 times, in which data from these experiments was pooled.

III. RESULTS AND DISCUSSION

Influences of UV light on skin cells have been established in many studies [14, 15]. As well as much evidence has reported that UV can cause DNA damage in melanocytes UV melanocytes[16, 17]. However, human lymphocytes have rarely been utilised to evaluate early and late cellular responses to UV irradiation. In order to estimate the initial cellular responses post UV irradiation, human lymphocytes were immediately subjected to viability test, comet assay, apoptotic and chromosomal analyses following UV exposure, at first generation post irradiation. The findings

demonstrated that UV irradiation significantly decrease ($p \leq 0.005$) growth of human lymphocytes at first population doubling following 5, 10, 15, 20 and 25 J/cm² irradiation as shown in figure 3, panel A. The response was in dose-dependent manner; higher doses gave higher responses (decrease in cell growth) than lower doses of UV irradiation. Moreover, early comet data showed a significant total DNA damage ($p \leq 0.005$) in irradiated cells (5, 10, 15, 20 and 25 J/cm²) compared to the control (0 J/cm²) at first population doubling following irradiation. (See figure 3, panel B). Data suggested that high DNA damage could instigate cell death in human lymphocytes following UV irradiation. In addition, chromosome aberrations were significantly ($p \leq 0.005$) observed in the irradiated experimental groups following 10, 15, 20 and 25 J/cm² UV irradiation at first generation compared to the control group. However, 5 J/cm² irradiated cells did not display initial chromosomal induction compared to the control, although this group demonstrated a high induction of apoptosis ($p \leq 0.005$). Similarly, other experimental groups (5, 10, 15, 20 and 25 J/cm²) showed significant levels of apoptosis compared with the control. Both early chromosomal and apoptotic responses of human lymphocytes were dose-dependent manner, which showed a linear apoptotic and chromosomal inductions to the UV irradiation doses as shown in figure 3, panel C. RapiV-Otrin and co-authors have stated that UV can cause DNA damage in human skin cells, which can be mediated by a functional connection between UV-damaged DNA binding protein complex, proteasomal degradation of p48 and chromatin remodeling during early step of nucleotide excision repair [18].

Furthermore, UV irradiation can significantly increase reactive oxygen species (ROS) generation and consequent DNA damage [19]. Oxidative stress may also play a role in DNA strand breaks following UV-B irradiation [20]. Data also suggested that the linearity in level of apoptosis could elucidate the linear responses in the cell death in the irradiated groups following UV irradiation.

Delayed cellular responses were measured after 12 population doublings post UV irradiation. The late data of viability test showed that 5 J/cm² dose was significantly able to decrease cell viability ($p \leq 0.01$) within the progeny of irradiated human lymphocytes compared to the control.

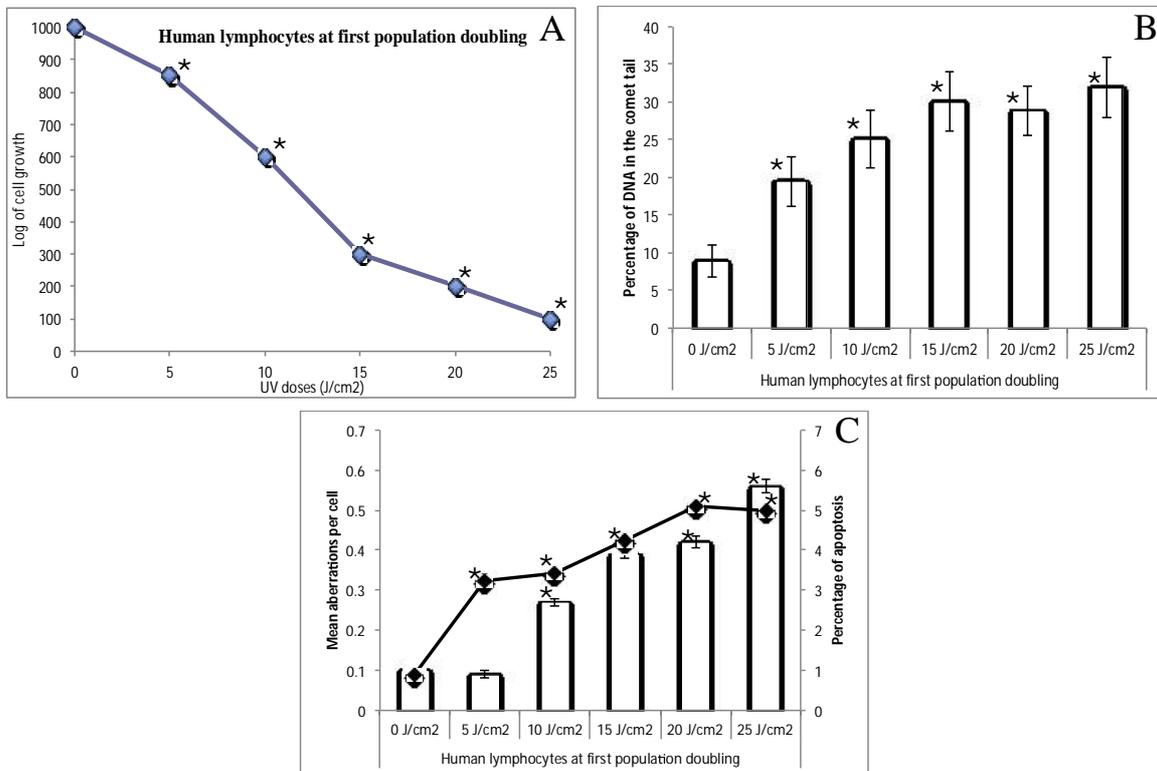
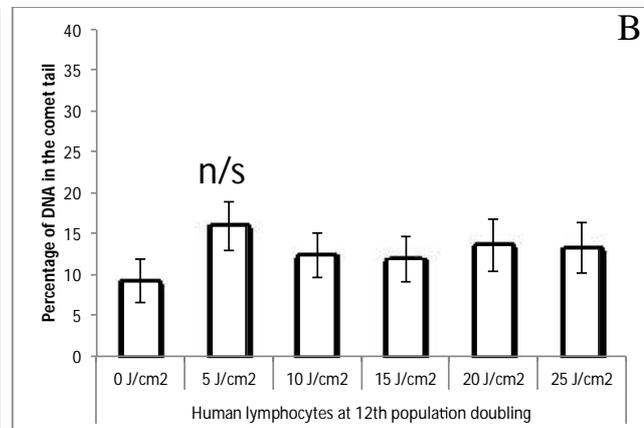
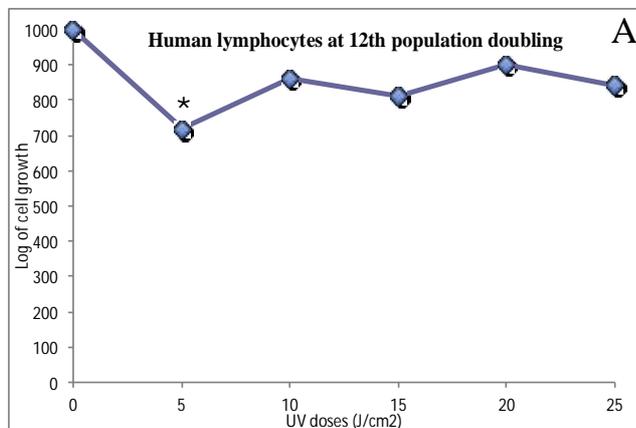


Fig. 3: Initial cellular responses in human lymphocytes following UV irradiation.

Cells were irradiated with 0, 5, 10, 15, 20 and 25 J/cm² UV-B. Cells then harvested for viability assay, comet assay, apoptotic and chromosomal analyses at first population doubling following irradiation. Panel A displays cell death post irradiation. Irradiated cells demonstrated a significant increase (*p≤0.005) in cell death compared to the control (0 J/cm²). The responses of irradiated cells to the irradiation were dose-dependent, in which higher doses gave higher levels of cell death. Panel B shows the total DNA damage in the comet tail within irradiated cells, which demonstrated significant responses (*p≤0.005) compared to the control. The data showed that the range of 5-25 J/cm² of UV irradiated can induce DNA damage within human lymphocytes. In panel C, columns represent the mean chromosomal aberrations; whilst lines represent the percentage of apoptosis in human lymphocytes. High induction of apoptosis (*p≤0.005) was observed in all the irradiated cells (5, 10, 15, 20 and 25 J/cm²). Irradiated cells also showed a significant chromosome damage (*p≤0.005) following 10, 15, 20 and 25 J/cm² UV irradiation. However, 5 J/cm² UV irradiation was not enough to induce chromosome aberrations in human lymphocytes, although a high induction of apoptosis was observed in these cells. Finding suggested that UV-B irradiation frequently induces cellular responses within human lymphocytes in terms of cell death, DNA damage, apoptosis and chromosome aberrations. However, cells with 5 J/cm² dose of UV irradiation do not show significant chromosomal damage.

Alternatively, other experimental groups did not show a significant increase in cell death after 12 generations following irradiation (See figure 4, panel A). Moreover, there was an elevation in the total DNA damage within the progeny of 5 J/cm² irradiated cells; however, it was statistically not significant. As well as the 10, 15, 20 and 25 J/cm² irradiated groups demonstrated insignificant DNA damage after 12 population doublings following UV irradiation compared to the control as shown in figure 4, panel B. Interestingly, the progeny of 5 J/cm² irradiated cell demonstrated significant apoptotic induction (p≤0.01); however, this group did not showed significant chromosomal instability compared to the control group (See figure 4, panel C). Data suggested that the high level of apoptosis eliminated the cells with high chromosomal damage. Conversely, the progeny of 10, 15, 20 and 25 J/cm² irradiated cells did not demonstrate induction of apoptosis; nevertheless, chromosomal instability was significantly observed (p≤0.005) in these progeny of these cells after 12 generations following UV irradiation. These results suggested there is an inverse relationship between apoptosis and chromosomal instability; high level of apoptosis frequently led to decrease in chromosomal instability in human lymphocytes and vice versa.

Interestingly, the response of delayed chromosomal damage was deviation from linearity, in which chromosomal instability in human lymphocytes was non-dose-dependent phenomenon. The experimental delayed data agreed with Laposaet *al.*, who documented that UV irradiation could cause long-term inhibition of p53, consequent DNA damage response and genomic instability [21]. UV irradiation can induce chromosomal instability through oxidative stress generating [22]. Furthermore, normal keratinocytes showed genomic instability following UV irradiation by measuring micronuclei. These cells displayed maintenance of micronuclei for long term. The study suggested that UV often contributes to the complex karyotype characteristic for human skin carcinomas [23].



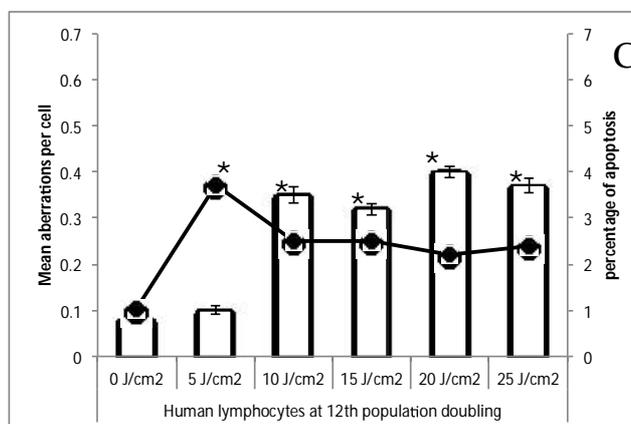


Fig. 4: Delayed damage responses within human lymphocytes after 12 generation post UV-B irradiation.

Cells were irradiated with a range of UV-B irradiation (0, 5, 10, 15, 20 and 25 J/cm²). Cells were then propagated until 12th population doubling to determine the delayed cellular responses within the progeny of irradiated cells. Panel A shows the rate of cell death within the progeny of irradiated cells. A significant decrease in cell viability (*p<0.01) was observed in the progeny of 5 J/cm² irradiated cells. Although, there was decrease in the cell viability in the progeny of other irradiated cells (10, 15, 20 and 25 J/cm²), but was statistically insignificant. Insignificant DNA damage was observed in the progeny of irradiated cells; although, there was an elevation in the percentage of DNA damage in 5 J/cm² irradiated group after 12 population doublings following irradiation as shown in panel B. In panel C, columns represent the mean chromosomal aberrations; whilst lines represent the percentage of apoptosis in human lymphocytes. High induction of apoptosis (*p<0.01) was observed in 5 J/cm² irradiated group after 12 population doublings following irradiation. However, these cells did not show a significant chromosomal instability. Alternatively, the progeny of other irradiated groups did not demonstrate significant apoptotic inductions, but chromosomal instability was significantly (*p<0.005) observed in these cells. Data suggested that exposure to higher than 5 J/cm² UV-B irradiation can lead to genomic instability in the human lymphocytes, which has been considered a hallmark of cancer induction. As well as data demonstrated an inverse relationship between apoptotic level and delayed chromosomal damage in human lymphocytes following UV irradiation.

The findings suggested that UV-B irradiation can induce early cellular responses in human lymphocytes following 5, 10, 15, 20 and 25 J/cm². As well as genomic instability can be observed in lymphocytes after 12 population doublings post 10, 15, 20 and 25 J/cm² UV irradiation in terms of chromosome aberrations. Alternatively, 5 J/cm² UV irradiation can cause a delayed induction of apoptosis, but not chromosomal instability in lymphocytes. Data also suggested that there is an inverse relationship between apoptosis and chromosomal instability.

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